J Wood Sci (2000) 46:45–51

ORIGINAL ARTICLE

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Immunogold labeling of an extracellular substance producing hydroxyl radicals in wood degraded by brown-rot fungus *Tyromyces palustris*

Received: March 26, 1998 / Accepted: April 19, 1999

Abstract A fraction containing low-molecular-weight peptides that catalyzes redox reactions between electron donors and O_2 to produce ·OH, was partially purified from wood-decaying cultures of the brown-rot fungus *Tyromyces palustris*. Polyclonal antibodies raised to the fraction were used for immunogold labeling of transverse sections of sapwood of spruce in various stages of degradation by *T. palustris* to demonstrate the cellular localization of the ·OH-producing substance. Initially, the wood cell wall was attacked primarily by fungal hyphae growing in the cell lumen. During the early stages of degradation, the gold label was localized in the fungal cytoplasm and cell wall and in the extracellular slime sheath surrounding the fungal cell wall. The gold label also was found throughout the wood cell wall, although the cell wall remained almost intact so long as the fungal hyphae remained in the lumen. Thus, the ·OH-producing substance is secreted by the hyphae into the lumen, and it diffuses through the S3 layer into the S2 layer and the middle lamella. The role of this ·OH-producing system in wood degradation by *T. palustris* is discussed.

Key words Brown-rot fungi · Immunogold labeling · Transmission electron mircoscopy · Wood decay · Hydroxyl radical

Introduction

Brown-rot fungi can degrade crystalline cellulose in wood, even though they lack exo-1,4-glucanase activity.[1,2] Although they also can degrade the lignin in wood, they preferentially remove the cellulose and hemicellulose, leaving an amorphous, brown, crumbly residue that is primarily lignin.[3,4] Nevertheless, brown-rot fungi degrade and metabolize crystalline cellulose only under conditions in which their ligninolytic systems are active. Furthermore, the cellulolytic systems of brown-rot fungi are always active when these organisms are degrading lignin.[4] The rates of ·OH-production in cultures of brown-rot fungi, including *Tyromyces palustris*, are directly proportional to the degradation rates of wood, crystalline cellulose, and lignin substructure model compounds in the fungal cultures.[5,6]

In the early stages of degradation by brown-rot fungi, the S2 layers of the wood cell walls are degraded extensively. The S3 layers adjacent to the lumens are less affected when they are attacked by fungal hyphae from the lumens.[7–9] During these early stages of degradation, fungal enzymes such as cellulases and peroxidases are too large to penetrate the cell walls.[10,11] Thus, brown-rot fungi have a unique degradative system that utilizes ·OH to degrade the wood cell wall at some distance from the hyphae in the lumen.

Extracellular substances that catalyze a one-electron oxidation reaction in the presence of H_2O_2 have been isolated from wood-degrading cultures of the brown-rot fungi *Gloeophyllum trabeum* and *T. palustris*.[12–14] The partially purified preparations contain Fe(II) (0.12%–0.2%), protein (12%–23%), and neutral carbohydrate (22%–35%). The molecular weights of these substances, as estimated by gel filtration, are quite small, between 1500 and 5000 daltons. The preparations catalyze a redox reaction between electron donors and O_2 to produce ·OH *via* O_2^- and H_2O_2. Furthermore, they reduce Fe(III) to Fe(II) and strongly bind Fe(II). Most of the ·OH in cultures of *T. palustris* is produced *via* redox reactions between O_2 and certain electron donors, catalyzed by this low-molecular-weight substance.[14] Thus, this substance appears to play an important role in the degradation of wood by *T. palustris*.

The object of the present study was to demonstrate the cellular localization of this ·OH-producing, low-molecular-weight substance from *T. palustris* during the fungal degradation of Japanese beech and spruce.

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Materials and methods

Organism

The brown-rot fungus *Tyromyces palustris* (Berk. et Curt) Murr. FRI 05707 was obtained from the Forestry and Forest Products Research Institute, Tsukuba, Ibaragi, Japan.

Culture conditions

Basal agar medium was prepared as described previously,[4] except that the medium contained 0.5% glucose and 10× concentrated trace elements. For extraction of the extracellular ·OH-producing substance, sterilized sawdust (2g dry weight) from Japanese beech with a distilled water content of 60% was sprinkled evenly over of the surface of the basal agar medium (30ml in 300-ml Erlenmeyer flasks). The sawdust was extracted twice with acetone and dried before use. The medium was inoculated with a small piece of *T. palustris* mycelial mat and incubated at 28°C in air.

For immunogold-labeling experiments, the basal agar medium (30ml in 300-ml Erlenmeyer flasks) was inoculated with a small piece of *T. palustris* mycelial mat and incubated at 28°C until the mycelia covered the surface of the medium. Wood pieces (approximately 2 × 2 × 0.5cm) from sapwood of spruce (*Picea jezoensis* Carr.) were extracted with acetone, sterilized with ethylene gas, and weighted. Three pieces were placed on each mycelial mat, and the cultures were incubated further at 28°C. At the indicated intervals the mycelia were removed carefully from the wood pieces using running water, and the wood pieces were dried and weighted. Control flasks were treated as above but lacked the fungal mycelia. Samples of decayed wood and controls were used for transmission electron microscopy (TEM).

Preparation of extracellular ·OH-producing fraction

After 30 days of incubation, 30ml of distilled water was added aseptically to each culture. The mixtures were stirred with a glass rod and centrifuged at 10000*g* for 20min at 4°C. The supernatants from 100 flasks were combined and lyophilized. The residue was dissolved in 100ml distilled water, and the precipitate was removed by filtration through filter paper. Acetone (−10°C) was added to the solution, to 70% (v/v). The solution was allowed to stand overnight at 4°C and then centrifuged. The resulting pellet was dissolved in 50ml distilled water, and 10ml was applied to a Sephadex G-50 column (Ø3.8 × 60cm), previously equilibrated with distilled water. The column was eluted with distilled water, and fractions (5.5ml) were collected. Fractions were assayed for ethylene production from 2-keto-thiomethylbutyric acid (KTBA) (see below) and measured for ultraviolet (UV) absorbance at 280nm. Fractions containing the major ethylene-generating peak were pooled, reduced to 10ml by lyophilization, and applied to a Sephadex G-25 column (Ø3.8 × 60cm) that had been equilibrated with distilled water. The column was eluted with distilled water, and fractions (5.5ml) were collected and assayed for ethylene generation. Again, fractions containing the major ethylene-generating peak were pooled and applied to a diethylaminoethyl (DEAE) Affi-gel blue gel column (Ø1.4 × 10cm) (Bio Rad) that was equilibrated with 0.02M Tris-HCl (pH 8.0). The column was eluted with a linear gradient of 0–1.4M NaCl. Fractions containing the major ethylene-generating peak were pooled and concentrated approximately fivefold by lyophilization. The concentrated solution was desalted on a Sephadex G-10 column, yielding 200mg from 100 flasks.

Ethylene production from KTBA

Ethylene production from KTBA was measured as described previously.[6] Reaction mixtures contained 300µl of sample, 300µl of 0.01M H_2O_2, 300µl of 0.16M Na acetate buffer (pH 4.5), and 50µl of 0.1M KTBA. After 1h at 30°C, 1ml of gas was removed from the headspace, and the ethylene concentration was measured directly by gas chromatography as previously described.[6]

Preparation of polyclonal antibody

The partially purified ethylene-generating substance was coupled to bovine serum albumin as the soluble carrier protein, using glutaraldehyde,[15] and emulsified with an equal volume of Freund's complete adjuvant. Japanese white rabbits were given subcutaneous injections (1ml). After 1 month the rabbits received subcutaneous injections of the conjugate emulsified with an equal volume of Freund's incomplete adjuvant (1ml). Four additional booster injections were given at 2-week intervals. The rabbit immunoglobulin G (IgG) fraction was purified by affinity chromatography using a DEAE Affi-gel blue gel (Ø1.4 × 10cm) (Bio Rad), coupled with the ·OH-generating fraction. The purified antibody and the preimmune control serum were preincubated with sound wood sawdust (2mg/ml) for 1h prior to use to remove nonspecific binding agents.[16]

Immunoblotting

The partially purified ·OH-generating fraction was separated by tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)[17] and blotted on polyvinylidene difluoride (PVDF) membranes by the method of Ploug et al.[18] The membranes were incubated for 1h with casein buffer [0.01M Tris-HCl (pH 7.6) containing 0.9% NaCl (w/v) and 0.5% casein (w/v)].[19] The membranes then were overlaid with either antibody or preimmune serum (diluted 1:400 in casein buffer) and incubated for 1h. After three washes with casein buffer the membranes were incubated for 1h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG diluted 1:5000 in casein buffer. Antibody binding was visualized using 3,3-diaminobenzidine, and the reaction was terminated by

washing in distilled water. All incubations were carried out at room temperature.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISAs) were conducted to investigate the effects of glutaraldehyde-paraformaldehyde fixation on the reaction between the ·OH-producing antigen and the antibody. Antigen (0.2 mg/ml) was serially diluted in phosphate-buffered saline (PBS) and used to coat 96-well microtiter plates. The plates were incubated for 2 h at room temperature with 0.2% glutaraldehyde and 2% paraformaldehyde in PIPES buffer (pH 7.4). After removing the liquid the wells were washed three times with casein buffer, blocked for 5 min with the same buffer, and treated with the primary antibody diluted 1:100 in casein buffer for 2 h at room temperature. This was followed by a 2 h incubation with HRP-conjugated goat anti-rabbit IgG diluted 1:5000 in casein buffer. The wells were rinsed thoroughly to remove any unbound conjugate, and each well was filled with 0.1 ml of 0.06% 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Color development was detected at 405 nm using an ELISA plate reader (Bio Rad model 2550).

Tissue preparation for TEM

Following incubation of the cultures, small pieces (1 mm^3) of earlywood were cut from the decayed spruce sapwood and from the sound spruce sapwood controls. The pieces were fixed with 0.2% glutaraldehyde and 2% paraformaldehyde in 0.1 M PIPES buffer (pH 7.4), with degassing for 2 h at 4°C. The samples were washed three times in 0.02 M Tris-HCl buffer (pH 7.6) for 10 min each, dehydrated in an ethanol series (50%–100%) for 30 min each, and substituted by London Resin White (hard medium) (London Resin Co.). The samples were transferred into gelatin capsules and polymerized at 60°C overnight. Sectioning was performed with a diamond knife on a Sorvall MT-6000. The ultrathin sections were collected on formval-coated nickel grids and examined with an Hitachi H-600 at 100 kV.

Immunogold staining

Ultrathin sections of decayed wood were quenched in casein buffer as a blocking agent[19] for 30 min and then incubated for 1 h in drops of primary antibody diluted in PBS. The sections were washed eight times for 3 min each in PBS and incubated for 1 h in drops of protein A-gold (15 nm) diluted in PBS. Following eight washes in PBS the sections were rinsed twice for 3 min with distilled water followed by 20 min of staining in 2% (w/v) aqueous uranyl acetate. All steps were carried out in a moist chamber at room temperature. In control experiments, decayed wood sections were (1) incubated with preimmune serum instead of the antibody; (2) incubated with antibody preadsorbed with the antigen; and (3) treated with protein A-gold without an initial incubation step. In an additional control experiment, sound wood sections were treated as above with the primary antibody.

Results

Partially purified ·OH-producing fraction

The low-molecular-weight, partially purified preparation catalyzed a redox reaction between an electron donor, such as NADH, and O_2 to produce ·OH *via* O_2^- and H_2O_2, as previously described.[13] It also reduced Fe(III) to Fe(II) and strongly adsorbed Fe(II), as previously described.[13] Tricine-SDS-PAGE of the ·OH-producing fraction, following Sephadex G-50 and DEAE Affi-gel blue (Fig. 1, lanes 1 and 2, respectively), revealed the presence of three peptide bands with molecular weights of 3.6, 7.2, and 10.5 kDa, respectively. The N-terminal sequences of these peptides exhibited a high degree of homology (data not shown).

Immunoreactions

Each of the three peptide bands from tricine-SDS-PAGE reacted on the immunoblot to the polyclonal antibody raised to the ·OH-producing fraction (Fig. 1, lane 3). In contrast, no reaction was observed when rabbit preimmune serum was used instead of the antibody (data not shown).

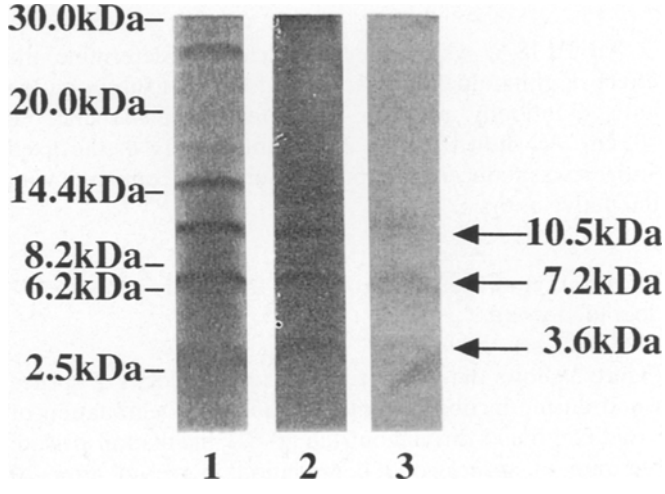

Fig. 1. Tricine sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and immunoblot analysis of the ·OH-producing fraction. The low-molecular-weight ·OH-producing fraction was partially purified from cultures of *Tyromyces palustris* and electrophoresed, as described in the text. Lane *1:* Tricine SDS-PAGE of the partially purified fraction from Sephadex G-50. Lane *2:* Tricine SDS-PAGE of the partially purified fraction following diethylaminoethyl (DEAE) affi-gel blue chromatography. Both gels were stained with silver nitrate. Lane *3:* The partially purified fraction was subjected to Tricine SDS-PAGE, electrotransferred to a polyvinylidene difluoride (PVDF) membrane, and reacted with polyclonal antibody, as described in the text

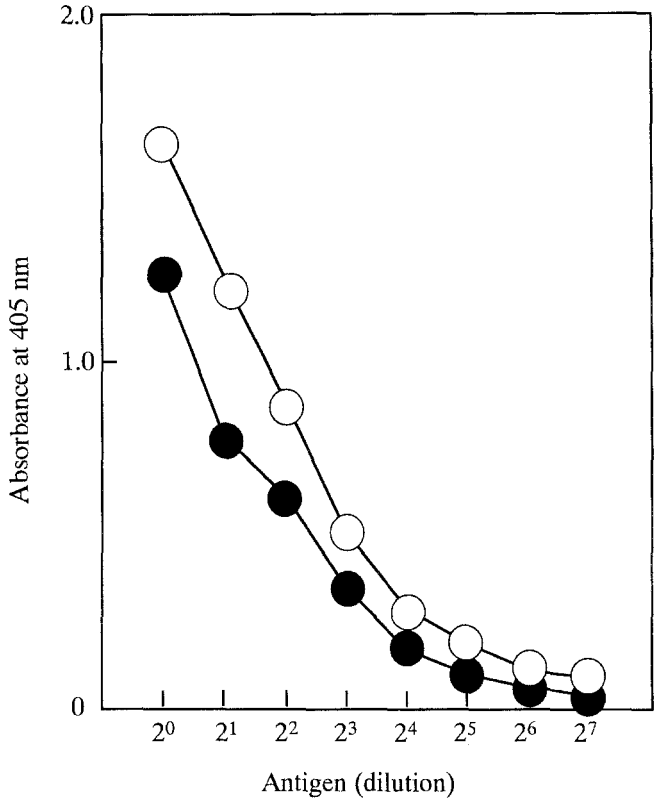

Fig. 2. Effect of glutaraldehyde-paraformaldehyde fixation on the reaction between the ·OH-producing fraction and the polyclonal antibody. Enzyme-linked immunosorbent assays (ELISAs) were performed as described in the text. *Open circles*, ·OH-producing fraction without fixation; *filled circles*, ·OH-producing fraction following fixation with 0.2% glutaraldehyde/2% paraformaldehyde

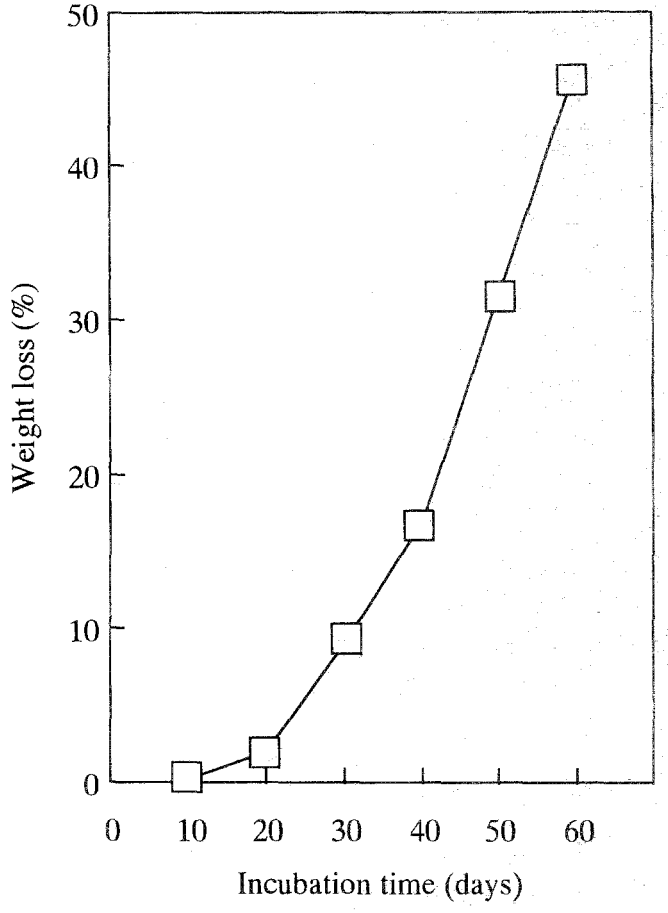

Fig. 3. Percent weight loss of sapwood following incubation with *T. palustris*. Blocks of spruce (*squares*) were incubated with the fungus, and the weight loss was determined as described in the text

An ELISA, which was conducted to determine the effect of glutaraldehyde-paraformaldehyde fixation on the antigen-antibody reaction, exhibited an absorbance at 405 nm. As shown in Fig. 2, the antigenicity of the fixed antigen was reduced by approximately 30% compared with the native antigen.

Localization of the ·OH-producing fraction in degraded wood

Figure 3 shows the percent weight loss in blocks of spruce wood during incubation with *T. palustris*. Degradation of wood continued throughout the 60-day incubation period; the rate of sprucewood degradation increased after 20 days.

Localization of the low-molecular-weight ·OH-producing fraction from *T. palustris* was observed using a polyclonal antibody followed by immunogold-labeling and TEM. Little nonspecific background labeling was observed in the ultrathin sections of sound softwood, decayed wood treated only with protein A-gold without an initial incubation step, decayed wood treated with preimmune serum instead of antibody, or decayed wood treated with antibody that had been preadsorbed with antigen (data not shown). After 10 days of incubation with spruce earlywood, the *T. palustris* hyphae in the lumen produced networks of slime-like material, and the hyphae were attached to the S3 layer of the wood cell wall by this network (Fig. 4a).

During the initial stages of degradation of spruce earlywood, gold labeling was observed in the periphery of the fungal cell wall, in the fungal cytoplasm, in the extracellular slime sheath, and in the fungal sheath network. Significantly, gold particles were present in the secondary wall and the middle lamella in the cell wall of the spruce earlywood, even though degradation was at an early stage (Fig. 4a,b). The extent of gold labeling in the cell corner was small compared to that within the secondary wall (Fig. 4b). After 40 days of incubation with *T. palustris*, the extent of gold labeling within the hyphae and the wood cell wall was relatively small (Fig. 4c). After 60 days of incubation with the fungus the hyphae and the cell walls of the wood were only lightly labeled (Fig. 4d). The thickness of some wood cell walls in which fungal hypha presented in the lumen changed little even when the weight loss became about 50%. The erosion or bore hole formation in the wood cell wall where fungal hypha in the lumen attached to the S3 layer were rarely observed.

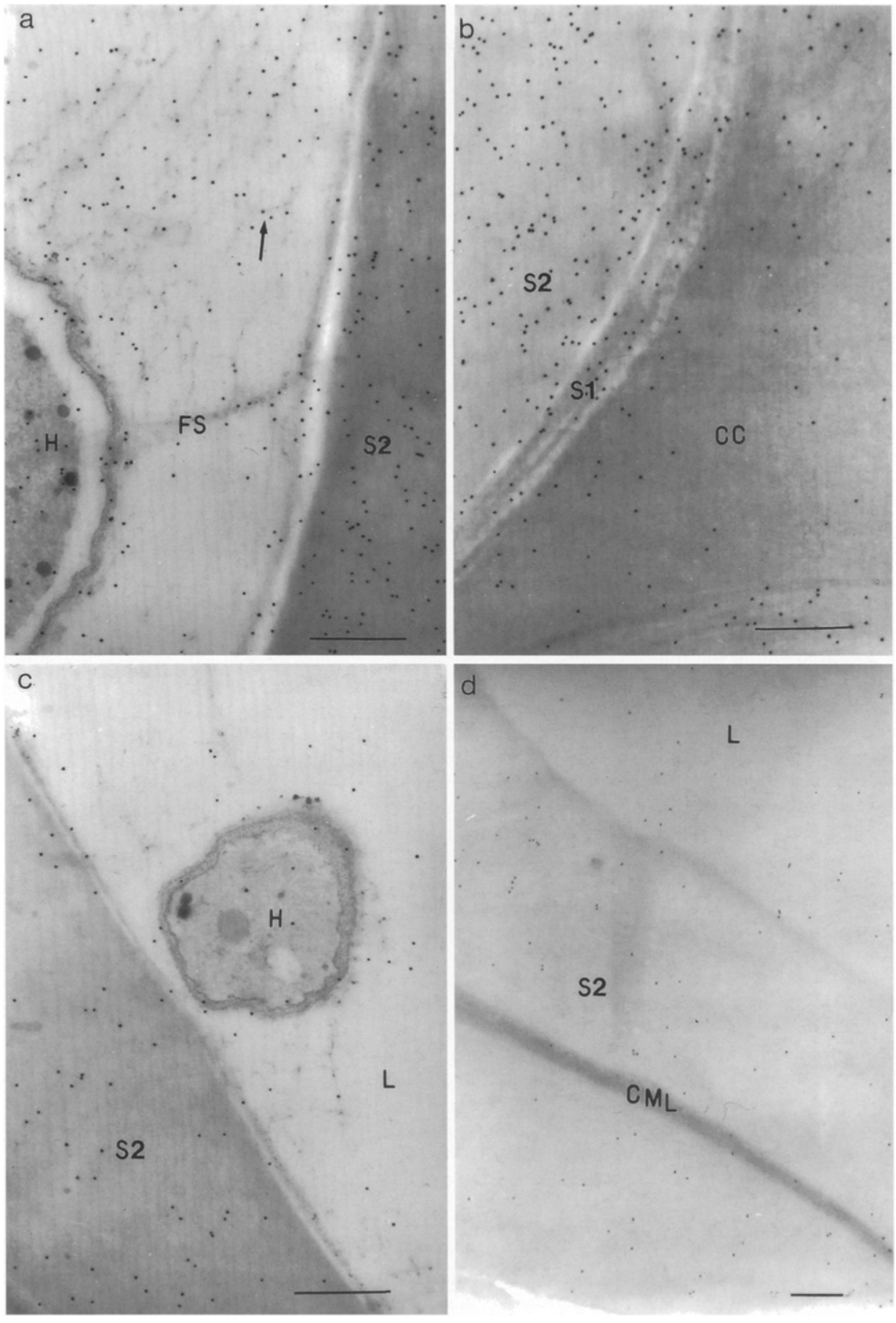

Fig. 4. Transmission electron micrographs of transverse sections of *T. palustris*-degraded spruce sapwood, showing the immunogold-labeled ·OH-producing substance. Labeling and transmission electron microscopy (TEM) were as described in the text. **a** Gold particles (*arrow*) in the hypha, fungal sheath, and secondary wall of the sprucewood after 10 days of incubation with *T. palustris*. **b** Gold particles in the sprucewood cell wall after 10 days of incubation with *T. palustris*. **c** Decreased immunogold labeling in the sprucewood cell wall and fungal hypha after 40 days of incubation. **d** Gold particles localized in the wood cell wall after 60 days of incubation. *H*, hypha; *FS*, fungal sheath; *S1*, S1 layer of the wood cell wall; *S2*, S2 layer of the wood cell wall; *CC*, cell corner; *CML*, compound middle lamella; *L*, lumen. Bars 0.5 μm

Discussion

Transmission electron microscopy has shown that the secondary walls of spruce wood are degraded by the brown-rot fungus *T. palustris* via a common mode of attack.[7–9] Progressive erosion of all cell wall components or penetration of the lumen surface by hyphae within the lumen is rarely observed; nevertheless, weight loss reached about 50%. Thus, wood decay by *T. palustris* does not require the hyphae to come in close contact with the substrates. The polysaccharides, mixed with lignin and embedded in the wood cell wall, are degraded easily at a distance from the hypha.[7–9] This phenomenon suggests that *T. palustris* produces an extracellular degradative agent capable of diffusing through the S3 layer into the S2 layer. The latter is the most susceptible to degradation in both hardwood and softwood.[7–9]

The results in Fig. 1 demonstrate isolation of a low-molecular-weight ·OH-producing, peptide fraction from *T. palustris* and production of polyclonal antibodies specific for this fraction. Immunogold labeling and TEM of ultrathin slices of sound wood and the controls with decayed wood (data not shown) demonstrate the specificity of the antibody and methods used here.

Figure 4 demonstrates that the ·OH-producing substance is localized within the fungal cytoplasm and cell wall, the extracellular sheath surrounding the fungal cell wall, and the cell walls of the decayed wood, even at early stages of degradation in which the weight loss of the wood is small (Fig. 3). The immunogold labeling of the ·OH-producing substance in wood decomposed by *T. palustris* varies in intensity with the stage of cell wall degradation: During the initial stages of wood degradation intense labeling occurs throughout the wood cell wall where modifications of the cellulose and lignin substructures are about to begin. During advanced stages of fungal degradation the labeling is weak, in both the fungal hyphae and the wood cell wall. In general, the S2 layer of the cell wall is attacked first, and the S3 layer and middle lamella remain partly intact, even during advanced stages of degradation, when the attack is initiated by the fungal hyphae in the lumen.[7–9] Thickness was not changed throughout the incubation time, but at advanced stages of fungal decay the changes of wood cell wall could be seen. During the initial stages of wood decay by *T. palustris*, the extracellular ·OH-generating substance, produced by the fungus, diffuses through the S3 layer of the wood cell wall and into the S2 and S1 layers and the middle lamella.

Brown-rot fungi produce endo-β-1,4-glucanase but lack exo-β-1,4-glucanase, which is indispensable for hydrolyzing crystalline cellulose and natural cellulose.[20] These fungi also lack phenol oxidases, such as lignin peroxidase, manganese peroxidase, and laccase, which play an important role in lignin degradation by white-rot fungi.[21,22] Furthermore, enzymes such as cellulases and peroxidases are too large to penetrate cell walls during the early stages of wood degradation by brown-rot fungi.[10,11] Nevertheless, brown-rot fungi, including *T. palustris*, degrade the cellulose in wood extensively and the lignin in wood to a lesser but significant degree during stages in which the fungi produce large amounts of ·OH.[4,5,23] Hydroxyl radicals can depolymerize natural cellulose to a chemical structure that is similar to brown-rotted cellulose.[24] Hydroxyl radicals also are known to depolymerize the cellulose in wood extensively, although they cannot remove the lignin in wood to a significant degree.[25] However, hydroxyl radicals can attack lignin-related compounds, causing a variety of chemical modifications including aromatic ring hydroxylation, ring opening, demethoxylation, hydroxylation, and oxidation of methyl groups.[26] A significant reduction in the degree of polymerization of the cellulose is typical of the early stages of wood degradation by brown-rot fungi.[1] In addition, the lamellar structure of the lignin in the wood cell wall is lost during decay by brown-rot fungi.[27] The rate of ·OH production in *T. palustris* cultures is correlated with the rate of degradation of wood and crystalline cellulose and lignin models in the cultures.[4] Furthermore, most of the ·OH produced in *T. palustris* cultures is generated by redox reactions between unknown electron donors and O_2, which are catalyzed by the low-molecular-weight substance.[14] Finally, pretreatment of wood with ·OH results in a marked increase in the amount of cellulose hydrolysis by purified endoglucanase from the brown-rot fungus *Poria placenta*. In fact, this cellulase degrades little if any microcrystalline cellulose unless the wood is pretreated with ·OH.[28]

Based on the results in this study and of previous work, we propose the following mechanism for wood degradation by the brown-rot fungus *T. palustris*: During the initial fungal attack on the wood cell wall, the low-molecular-weight substance is secreted by hyphae into the lumen. This effector is able to reduce the Fe(III) in wood to Fe(II), chelate the Fe(II), and penetrate or diffuse into the wood cell wall. With the Fe(II), the effector catalyzes a redox reaction between an electron donor and O_2 to produce ·OH *via* O_2^- and H_2O_2. The ·OH produced in the wood cell wall attacks chemical constituents of the cell wall and causes the depolymerization of cellulose, both crystalline and noncrystalline, and modification of the lignin. After that, enzymes such as endoglucanases can penetrate the cell wall and act on the cellulose and hemicellulose.

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